

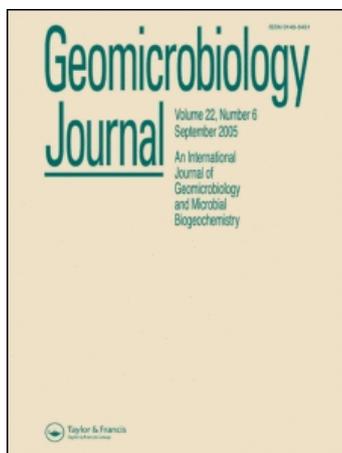
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### ***Dunaliella* Cells in Fluid Inclusions in Halite: Significance for Long-term Survival of Prokaryotes**

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# *Dunaliella* Cells in Fluid Inclusions in Halite: Significance for Long-term Survival of Prokaryotes

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A 90-m-long (100,000 year old) salt core from Death Valley, California, contains cells of the algal genus *Dunaliella* co-trapped with prokaryote cells in fluid inclusions in halite. It is hypothesized that *Dunaliella* cells provided glycerol, the carbon source needed by halophilic *Archaea* for survival over periods of tens of thousands of years. Support for this hypothesis includes: observations that intracellular materials leaked from *Dunaliella* cells into fluid inclusions; the distribution of *Dunaliella* cells in the Death Valley core, which matches the distribution of culturable prokaryotic cells; and halophilic *Archaea* cultured from the Death Valley core grew in media containing glycerol as the only carbon source.

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**Keywords**  $\beta$ -carotene, *Dunaliella*, Death Valley, fluid inclusions, glycerol, halite, Saline Valley, survival

## INTRODUCTION

### The Halophilic Alga *Dunaliella*

Species of the algal genus *Dunaliella* (Chlorophyta) are well known to inhabit hypersaline lakes and lagoons on all continents (Ginzburg 1987; Borowitzka and Borowitzka 1988; Oren 2005) and are commonly the only eukaryotic algae found in extremely saline lakes (Borowitzka 1981). *Dunaliella* cells are able to maintain osmotic equilibrium with concentrated brines through accumulation of intracellular glycerol at concentrations up to 6–7 M (Avron 1992; Elevi Bardavid et al. 2008).

*Dunaliella* cells do not contain a regular cell wall but instead have an elastic mucous surface coat (Ben-Amotz 1999) called glycocalyx. The lack of a rigid cell wall allows *Dunaliella*

species to exhibit a range of forms, even under optimal growth conditions. Vegetative cells of *Dunaliella* are typically motile and biflagellate with pyriform (pear-shaped), lageniform (flask-shaped), fusiform (spindle-shaped), ellipsoid, ovoid, and spherical to subspherical shapes (Leonardi and Cáceres 1997; Borowitzka and Siva 2007). Internal cell structures visible under a light microscope include a green chloroplast with cup, dish, or bell shape that contains a pyrenoid (Borowitzka and Siva 2007). *D. salina* cells contain pigments associated with their photosynthetic apparatus and thus exhibit chlorophyll autofluorescence at about 590 nm with especially bright areas associated with the chloroplast and pyrenoid bodies (Raja et al. 2007).

In general, *Dunaliella* morphologies depend on environmental conditions, stress, and stage of reproduction (Borowitzka 1981), which can make identification of *Dunaliella* species extremely difficult. Under optimal growth conditions, *Dunaliella* cells are typically green from chlorophyll pigments in their chloroplasts. When subjected to stressed conditions such as high light, high salinity, and/or low nutrient conditions, vegetative cells of some species, for example *D. salina* and *D. bardawil*, over-produce carotenoids, especially  $\beta$ -carotene (Ben-Amotz and Avron 1982; Loeblich 1982; Borowitzka and Borowitzka 1989; Ben-Amotz 1996, 1999; Del Campo et al. 2007). These vegetative orange cells may be flagellated or they may form round cysts called “aplanospores,” which develop a thick, rough wall and granular cytoplasm (Loeblich 1969; Borowitzka and Huisman 1993; Leonardi and Cáceres 1997; Ben-Amotz 1999).

Some *Dunaliella* species, under non-ideal conditions, such as low or high salinity or absence of nutrients, form round, deflagellated cells with a thick mucilaginous outer layer called a palmelloid (Leonardi and Cáceres 1997; Borowitzka and Siva 2007). When entering the palmelloid stage, *Dunaliella* cells lose their flagella and eyespot, if present (Leonardi and Cáceres 1997; Borowitzka and Siva 2007). In addition to the development of aplanospore and palmelloid stages, some *Dunaliella* species may produce zygospores when exposed to environmental stress (Hamburger 1905; Lerche 1937; Oren et al. 1995). The thick cell wall and orange color of zygospores resemble aplanospores.

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## Prokaryotes in Fluid Inclusions in Halite

Fluid inclusions in halite contain concentrated brines that are trapped during crystallization (“primary”) or along later healed fractures (“secondary”). Their size (sub-micrometer to millimeter scale), shape (cubic, rectangular prism, or irregular), number (up to millions in a single crystal), and orientation depend on the depositional environment and crystal growth rate (Roedder 1984; Lowenstein and Brennan 2001). Previous work on laboratory-crystallized halite and natural, surficial and buried halite demonstrated that prokaryotes are readily trapped and preserved in fluid inclusions (Norton and Grant 1988; Fredrickson et al. 1997; Mormile et al. 2003; Fendrihan and Stan-Lotter 2004; Adamski et al. 2006; Fendrihan et al. 2006; Lowenstein 2008; Schubert et al. 2009a). Studies also showed that prokaryotes can remain viable in fluid inclusions in halite for up to hundreds of millions of years (Reiser and Tasch 1960; Dombrowski 1963; Grant et al. 1998; Norton et al. 1993; McGenity et al. 2000; Stan-Lotter et al. 1999, 2002; Mormile et al. 2003; Gruber et al. 2004; Vreeland et al. 2000, 2007; Schubert et al. 2009b). Although prokaryotes occur in fluid inclusions in ancient halite as miniaturized, coccoid-shaped cells (Schubert et al. 2009a), little is known about the metabolism of these microorganisms over periods of thousands to millions of years.

A number of mechanisms that account for the metabolic state of prokaryotes over geological time scales have been suggested. Spore-forming bacteria, dormant cells in which no measurable metabolic activity or DNA repair occurs, were cultured from 250 million year old halite (Vreeland et al. 2000). Research on survival of prokaryotes in permafrost, however, shows that metabolically active cells capable of DNA repair may be better suited for long-term survival than dormant cells (Johnson et al. 2007). Prokaryotes that remain metabolically active in a fluid inclusion for protracted periods require an *in situ* energy source.

It has long been suggested that glycerol, first accumulated by, and then leaked from, *Dunaliella* cells, is a potential energy source for prokaryotes in saline lakes (Gochnauer et al. 1972; Rodríguez-Valera et al. 1980; Borowitzka 1981; Javor 1984; Oren 1993a, 1993b, 1995; Rawal et al. 1988; Elevi Bardavid et al. 2008; Falb et al. 2008). Here we document the coexistence of *Dunaliella* cells and prokaryote cells in fluid inclusions in ancient halite from the subsurface of Death Valley, California. We hypothesize that glycerol and other metabolites from *Dunaliella* cells that were trapped with prokaryote cells in fluid inclusions may have been available as energy and carbon sources for prokaryotes to maintain metabolic processes, including DNA repair, for periods of at least 100,000 years (Schubert et al. 2009c). Support for this hypothesis includes: (1) observations of intracellular material, such as  $\beta$ -carotene crystals, extruded from *Dunaliella* cells in fluid inclusions from the Death Valley core, (2) the distribution of *Dunaliella* cells in the Death Valley core, which closely matches the distribution of culturable prokaryotic cells, and (3) halophilic *Archaea*, cultured from the Death Valley core, grew in media containing glycerol as the sole carbon source.

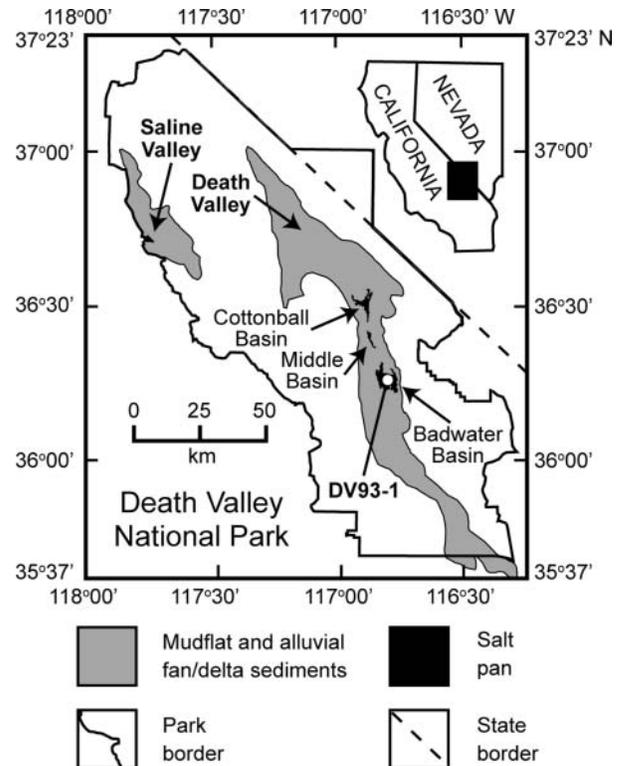


FIG. 1. Map of Death Valley National Park, including Death Valley and Saline Valley, California. The Death Valley core (DV93-1) was drilled in the middle of Badwater Basin. Modified from Schubert et al. (2009).

## GEOLOGIC BACKGROUND

### Modern Saline Valley

Modern halite samples used for this study were collected in Saline Valley, a closed basin in Death Valley National Park, eastern California (Figure 1). Saline Valley contains a normally dry saline pan,  $\sim 10 \text{ km}^2$ , fed by perennial groundwaters (Hardie 1968). The saline pan is underlain by halite and may be partially covered by a shallow brine pool during wet periods.

### Death Valley Core

Death Valley, a narrow, closed basin in Death Valley National Park, contains three sub-basins: Cottonball Basin, Middle Basin, and Badwater Basin (Figure 1). Ancient, subsurface halite samples used in this study were selected from a core drilled in Badwater Basin in 1993 (Figure 1). The top 90 m of the core records the last 100,000 years of salt and mud deposition in Death Valley (Li et al. 1996; Ku et al. 1998; Lowenstein et al. 1999) (Figure 2).

The Death Valley core, from depths of 7.7 to 18.0 m (10,000–35,000 years old), contains bedded halite with textures diagnostic of crystallization at the bottom of a perennial, hypersaline lake (Li et al. 1996). These textures include two types of vertically-oriented crystals: chevron halite and clear bottom growth halite. Chevron halite appears cloudy due to numerous

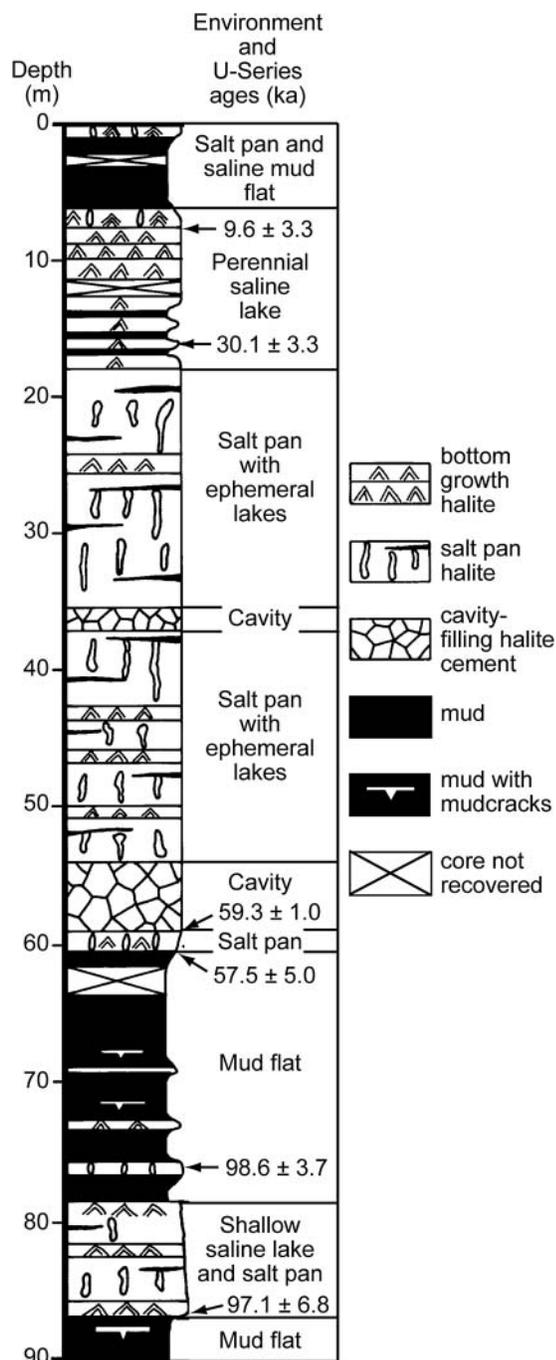


FIG. 2. Stratigraphic column of the Death Valley core from 0–90 m (0–100,000 years old) with U-series ages, interpreted paleoenvironments, and general halite textures (modified from Lowenstein et al. 1999).

fluid inclusions aligned along sub-millimeter-scale bands, rich and poor in fluid inclusions, whereas clear bottom growth halite contains fewer fluid inclusions.

From depths of 18 to 61 m (35,000–60,000 years old) and 78 to 87 m (~100,000 years old), the core contains features diagnostic of deposition in salt pan environments (Li et al. 1996).

Salt pan environments are typically dry, but ephemeral saline lakes may develop during wet periods. Numerous syndepositionary dissolution features characterize salt pan halite. Voids in porous salt pan halite crusts are commonly cemented by crystalline halite formed from saline groundwaters (Lowenstein and Hardie 1985). Chevron halite layers within salt pan intervals indicate crystallization from shallow saline lakes.

## MATERIALS AND METHODS

### Microscopy

Thin sections from the Death Valley salt core (5 x 7.5 cm) were studied using a Leica Wild M3Z stereomicroscope to document halite textures for paleoenvironmental interpretations. Cells of prokaryotes and *Dunaliella* were studied in wet mounts and in fluid inclusions in halite from thin sections and cleavage fragments under transmitted light at 100–1000x magnification on a Zeiss AXIO Imager.A1 microscope with an AxioCam MRm B&W camera and AxioVision software. An oil immersion objective (PLAN APO 100x/1.4 OIL) facilitated detailed study of *Dunaliella* cell morphologies and intracellular structures. Epifluorescence microscopy to examine autofluorescence of *Dunaliella* cells was done on the Zeiss AXIO Imager.A1 microscope mounted with an HBO 100 mercury lamp and Chroma Technology Corp. TRITC filter (exciter HQ545/30 nm, emitter HQ620/60 nm, beamsplitter Q570lp).

### Growth and Desiccation of *D. salina* and *D. bardawil* in Laboratory Cultures

*D. salina* Duna001 and *D. bardawil* UTEX LB2538 were grown photoautotrophically in batch cultures in 1.0 M NaCl medium (Pick et al., 1986) in the presence of 25 mM NaHCO<sub>3</sub> as a supplemental inorganic carbon source. Cultures were incubated at room temperature in Erlenmeyer flasks at a light intensity of about 50 μmol photons m<sup>-2</sup> s<sup>-1</sup>. The saline medium used for the batch cultures was not shaken, and slowly desiccated over the course of two to three months, leaving a single, centimeter-scale halite crystal in each container.

### Identification of Carotenoid Crystals

Orange, red, and pink crystals, 1–5 μm in length, were extracted from fluid inclusions in a halite crystal from 15.7 m (29,000 years old) in the Death Valley core. These suspected carotenoid crystals, including probable β-carotene, were studied using a JEOL 8900 Electron Microprobe to determine their chemical composition. To prepare a sample for analysis, a halite crystal was placed on a clean, glass microscope slide and the following steps (dissolution, de-salting) were performed. The halite crystal was slowly dissolved in 18.2 megohm water with less than 1 ppb total organic carbon (NANOpure DIamond UV Model #D11911 with Organic Free Deionized Feed kit #D50281) until the fluid inclusion containing the pigmented crystals was breached and the contents were released onto the microscope slide.

The salty water was then pipetted from the microscope slide and the remaining portions of the halite crystal were removed. The slide was gently washed with water several times to remove salts. Double-sided carbon tape on a 25 mm aluminum stub was gently pressed to the microscope slide to affix some of the pigmented crystals to the carbon tape. Observation using a Leica Wild M3Z stereomicroscope verified that the pigmented crystals were affixed to the tape. The crystals on the stub were then spot analyzed in the electron microprobe at 15.0 kV with a beam current of  $3.3 \times 10^{-8}$  A.

Similar orange, red, and pink crystals from a fluid inclusion in a halite crystal (0.1334 g) from 14.5 m (26,000 years old) in the Death Valley core were studied to determine their solubility in acetone. The halite crystal was submerged overnight in 100% ethanol, dried, and placed in a 1.7 ml microcentrifuge tube. The halite was then dissolved in 1.5 ml of 18.2 megohm water (described here). The sample was centrifuged at 13,400 rpm for 1 minute and the liquid was removed with a pipette. The pelleted contents were desalted by washing in water five times. After the final rinse, 20  $\mu$ l of 18.2 megohm water was added to re-suspend the pellet. The contents were pipetted onto a clean microscope slide and examined microscopically for orange, red, or pink crystals. The slide was then flooded with acetone while under microscopic observation. Photomicrographs (not shown) documented dissolution of the pigmented crystals in acetone.

Additional red, orange, and pink crystals in several fluid inclusions in halite crystals from 14.5 m in the Death Valley core were studied to determine their melting temperatures using a TMS 94 Linkam heating stage (Linkam Scientific Instruments Ltd, Surrey, England). Halite cleavage fragments that contained the pigmented crystals in fluid inclusions were mounted to glass cover slips and inserted into the heating stage. Under microscopic observation (Leitz Ortholux, Rockleigh, NJ), the stage was heated rapidly to 130°C and then slowly at 1.0°C per minute from 130.0–185.0°C. Photomicrographs (not shown) documented the melting points of various pigmented crystals in fluid inclusions. The melting points were compared with the results of Zscheile and White Jr. (1940).

## RESULTS

### *Dunaliella* cells in Fluid Inclusions in Halite

We studied *Dunaliella* cells from desiccated, uni-algal laboratory cultures to document their appearance and condition in fluid inclusions in halite and on halite surfaces. Wet mounts prepared from Saline Valley brines show the variety of *Dunaliella* cell morphologies in a modern, hypersaline environment. Halite crystals in salt crusts formed from Saline Valley surface brines document contemporaneous trapping of cells of *Dunaliella* and prokaryotes in fluid inclusions. Fluid inclusions in halite from the Death Valley core demonstrate the past association of *Dunaliella* cells and prokaryote cells in ancient saline lakes.

*Dunaliella* cells in desiccated, uni-algal, laboratory cultures. *D. salina* Duna001, a recent isolate from the North Arm of the Great Salt Lake in Utah, USA, and *D. bardawil* UTEX LB2538 were used to study cells in fluid inclusions from laboratory samples. Algal batch cultures desiccated slowly over the course of 2–3 months, leaving a single halite crystal in each container. Cells of *D. salina* and *D. bardawil* occurred in fluid inclusions and on the surfaces of the residual halite crystals (Figure 3). Cells of both strains showed pyriform, spherical to subspherical, or ellipsoid morphologies (Figure 3). *D. salina* cells were 8–15  $\mu$ m in length and typically green in color (Figure 3A–E); *D. bardawil* were 10–20  $\mu$ m in length and many were pigmented orange (Figure 3F–H). Some cells were colorless suggesting that they had bleached and lost all photosynthetic function (Figure 3H).

No matter what their color, all *D. salina* and *D. bardawil* cells were non-motile and appeared to lack flagella. Internal structures, including the chloroplast and pyrenoid, were readily observed in some cells (Figure 3A, F, and H). Damaged green and orange cells, in various stages of disintegration were also common (Figure 3E–H). Some degenerated orange cells contained internal globular structures; similar vesicular structures leaked outside *Dunaliella* cells (Figure 3F–H).

*Dunaliella* cells in environmental samples: Saline Valley. Pink surface brine (1 cm water depth, 31% salinity, 28°C) collected from Saline Valley in October 2005, was stored in a 125 ml plastic bottle (screw cap tight) in indirect sunlight at room temperature. Cells of prokaryotes and *Dunaliella* were observed in wet mounts from this brine 28–34 months after collection (Figure 4). Rod-shapes (1–10  $\mu$ m long) and coccoid shapes (1–2  $\mu$ m diameter) characterized the prokaryote community (Figures 4A–B). Culturing and PCR results indicate that halotolerant and halophilic *Archaea* and *Bacteria* populated the brine (unpublished data). *Dunaliella* cells observed in wet mounts were pyriform, spherical to subspherical, or ellipsoid in shape, and 4–16  $\mu$ m in length. Green *Dunaliella* cells could not be classified by species, but rare, motile, orange cells exhibited features identical to those reported for *D. salina* (reviewed by Oren 2005). Some cells were colorless indicating that they were bleached and no longer alive. The morphologies of motile and non-motile *Dunaliella* cells differed. Motile *Dunaliella* cells had ellipsoid and pyriform morphologies with two flagella, whereas non-motile cells were spherical to subspherical in shape and lacked flagella (Figure 4).

Some spherical *Dunaliella* cells, 5–15  $\mu$ m in diameter, contained thick cell walls suggestive of aplanospores or zygospores (Figure 4B, D, and E). *Dunaliella* cells contained one large, green or colorless, cup-shaped chloroplast (Figure 4C), some containing a pyrenoid, or had mottled interiors that lacked distinguishable structures (Figure 4G–I). The chloroplasts of cells showed typical chlorophyll autofluorescence using epifluorescence microscopy (TRITC filter) (Figure 5). In addition, bright autofluorescent vesicles were observed in the cytoplasm of some cells, which indicates storage of

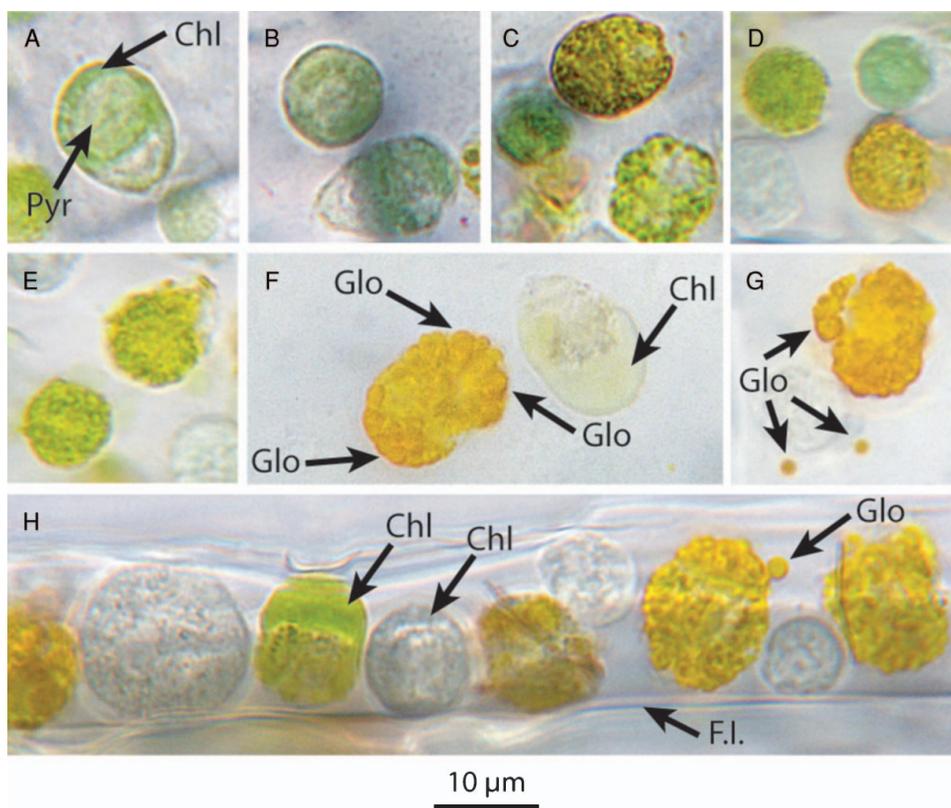


FIG. 3. Color photomicrographs of *D. salina* (Duna001) and *D. bardawil* (UTEX LB2538) cells in fluid inclusions and on halite surfaces from desiccated cultures. (A–E) *D. salina* cells in fluid inclusions in halite. Fluid inclusion boundaries are outside the field of view. Cells are green, orange, or colorless. The chloroplast (Chl) and pyrenoid (Pyr) are labeled in A. (F–G) *D. bardawil* cells on the surface of a halite crystal. Note the chloroplast (Chl) in F and orange globular structures (Glo) bulging from the plasma membrane in F and G. Two orange globules are not attached to the *Dunaliella* cell in G. (H) *D. bardawil* cells in fluid inclusions in halite (note the fluid inclusion boundary – F.I.). *D. bardawil* cells are orange, green, or colorless. Note that chloroplasts (Chl) may be green or colorless. An orange globule (Glo) is bulging from the plasma membrane of one cell.

autofluorescent material in vacuoles within the cytoplasm (Figure 5).

Halite crystals that were in direct contact with the brines from Saline Valley described above (collected in October 2005) contained cells of prokaryotes and *Dunaliella* in fluid inclusions (Figure 6). *Dunaliella* cells included ellipsoid ( $\sim 5 \times 10 \mu\text{m}$ ) (Figure 6A) and spherical to subspherical morphologies (Figure 6B–D). Clusters of small (3–5  $\mu\text{m}$ ), spherical to subspherical, green cells surrounded by mucilage, diagnostic of the palmelloid stage (Borowitzka and Siva 2007) were also observed (Figure 6C). One fluid inclusion contained a *Dunaliella* cell with pyriform shape and an intact flagellum (Figure 6E). No *Dunaliella* cells in fluid inclusions, including the one with the flagellum, were motile. Some *Dunaliella* cells had a well-defined, autofluorescent chloroplast containing chlorophyll (Figure 7).

*Dunaliella* cells in fluid inclusions in ancient halite: Death Valley core. Cells of prokaryotes and *Dunaliella* occurred together in fluid inclusions in ancient halite from the Death Valley core (Figure 8). Prokaryote cells exhibited coccoid (<1  $\mu\text{m}$ ) and rod shapes (1–2  $\mu\text{m}$  long) (Figure 8). Here we

describe the morphological features of ancient *Dunaliella* cells in fluid inclusions and compare them to modern *Dunaliella* cells.

*Dunaliella* cells in fluid inclusions in halite from the Death Valley core typically were 5–15  $\mu\text{m}$  in length (Figure 9; compare to Figures 3, 4, and 6). Ancient *Dunaliella* had spherical to subspherical shapes (Figure 9), but some had pyriform morphology (Figure 9H; compare to Figures 3E and 6E), and all lacked flagella. Algal morphologies did not systematically differ throughout the core. *Dunaliella* cells were typically colorless (Figure 9), but rare, yellow-green to yellow-orange cells, similar in color to modern *Dunaliella* cells, were also observed (Figure 11L–O; compare to Figure 3). The green and orange coloration of ancient *Dunaliella* cells strongly suggests that they still contained intact pigment molecules such as chlorophyll and carotenoids.

The glycocalyx of ancient *Dunaliella* cells was either of non-uniform thickness (micron-scale) (Figures 8, 9A, and 10A; compare to Figure 4D–E) or constant thickness (Figure 9D; compare to Figure 4B) and, as expected, had weak or no autofluorescence (Figure 10A). A cup-shaped chloroplast, observed in some *Dunaliella* cells (Figure 9B, E–G, and J–L;

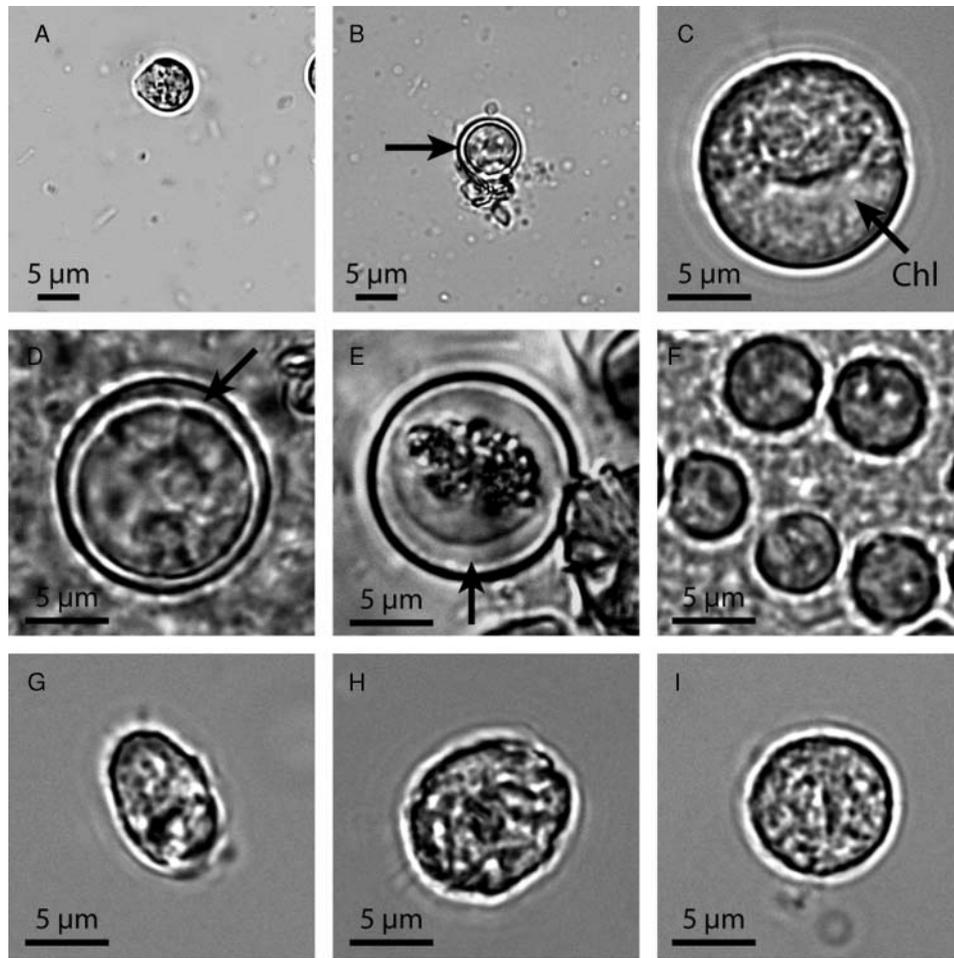


FIG. 4. Black and white photomicrographs of wet mounts showing prokaryote cells and *Dunaliella* cells from brines collected from Saline Valley, October 2005, observed 28–34 months after collection. (A) Pyriform *Dunaliella* cell with rod and coccoid prokaryote cells. (B) Spherical *Dunaliella* cell with glycocalyx of uniform thickness (arrow). Note abundant prokaryotes in the brine. (C) Spherical cell with chloroplast (Chl). (D–E) Spherical *Dunaliella* cells showing glycoprotein coats of non-uniform thickness for each cell (arrow). (F) Spherical, green *Dunaliella* cells. (G–I) *Dunaliella* cells with ellipsoid, subspherical, and spherical morphologies and no distinguishable internal structures.

compare to Figure 3H), was commonly autofluorescent (Figure 10B–D; compare to Figure 7A–C). The age of the samples (10,000–100,000 years old) apparently did not affect the autofluorescence intensity of the chloroplast of many cells (compare exposure times for Figure 10). In other cells, the chloroplast was poorly defined (Figure 9E; compare to Figures 3C and 4H). Many *Dunaliella* cells had mottled interiors and no distinguishable structures (Figure 9I; compare to Figures 3D–E and 4I).

#### Distribution of *Dunaliella* Cells in the Death Valley Core

Microscopic study of thin sections and halite cleavage fragments from 36 stratigraphic intervals in the Death Valley core confirmed the halite texture (clear bottom growth, chevron, cement) and paleodepositional environment (perennial saline lake, shallow saline lake, or groundwater) (Table 1). Sixteen layers studied contained clear bottom growth and chevron textures

from a perennial saline lake interval, 7 layers contained chevron textures from shallow saline lake intervals, and 13 layers contained cements that crystallized from hypersaline groundwaters (Table 1).

*Dunaliella* cells were heterogeneously distributed in the Death Valley core. Thirteen of the 16 halite layers examined from the perennial saline lake interval (8.1–18.0 m; 10,000–34,000 years old) contained *Dunaliella* cells in fluid inclusions (Table 1). In contrast, only one out of seven halite layers from interpreted shallow saline lake intervals contained cells of *Dunaliella* cells, and none of the 13 halite cement layers interpreted to have formed from groundwaters, contained *Dunaliella* cells (Table 1). The distribution of *Dunaliella* cells in the Death Valley core is consistent with the distribution of prokaryote cells and cultured strains (Table 1). Cells of prokaryotes and of *Dunaliella* were observed almost exclusively in halite from the perennial saline lake interval (Table 1). This indicates that

TABLE 1  
Distribution and relative abundance of *Dunaliella* cells in fluid inclusions in halite from the Death Valley core

Halite texture	Depth (m)	Age (ka)	<i>Dunaliella</i> /prokaryotes observed <sup>a</sup>	Prokaryotes cultured <sup>b</sup>
<i>Perennial saline lake</i>				
Chevron	8.1	10	+/+	—
Chevron	8.2	11	-/-	N.S.
Chevron	8.3	11	-/-	N.S.
Clear bottom growth	8.6	11	+/+	N.S.
Chevron	8.7	12	+/+	N.S.
Clear bottom growth	12.9	22	+/+	—
Clear bottom growth	13.0	22	+/+	+
Clear bottom growth	14.1	25	+/+	+
Clear bottom growth	14.2	25	+/+	N.S.
Clear bottom growth	14.5	26	+/N.S.	N.S.
Clear bottom growth	15.7	29	+/+	—
Clear bottom growth	16.5	31	+/+	—
Clear bottom growth	16.6	31	+/+	N.S.
Clear bottom growth	16.7	31	+/+	—
Clear bottom growth	17.8	34	+/+	++++
Clear bottom growth	17.9	34	N.S./N.S.	++ <sup>c</sup>
Clear bottom growth	18.0	34	+/+	—
<i>Shallow saline lakes</i>				
Chevron	50.8	55	-/-	—
Chevron	51.7	56	-/+	—
Chevron	82.8	100	-/-	—
Chevron	83.9	100	-/-	—
Chevron	84.9	100	-/-	—
Chevron	85.7	100	+/+	+ <sup>d</sup>
Chevron	86.7	100	-/-	—
<i>Groundwater</i>				
Cement	35.6	44	-/-	N.S.
Cement	43.8	50	-/-	N.S.
Cement	50.3	55	-/-	—
Cement	56.3	58	-/-	N.S.
Cement	57.3	58	-/+	N.S.
Cement	58.0	58	-/-	N.S.
Cement	58.5	58	-/-	—
Cement	60.1	58	-/-	—
Cement	60.4	58	-/-	—
Cement	79.2	100	-/-	—
Cement	81.5	100	-/-	—
Cement	84.4	100	-/-	—
Cement	86.7	100	-/-	—

<sup>a</sup> + = *Dunaliella* cells or prokaryotes observed in fluid inclusions in halite from this depth; — = *Dunaliella* cells or prokaryotes not observed in fluid inclusions in halite from this depth. Data for prokaryotes from Schubert et al. (2009 and in review).

<sup>b</sup> + = one strain cultured from this depth; ++ = two strains cultured from this depth; +++ = four strains cultured from this depth; — = zero strains cultured from this depth. Data from Schubert et al. (in review) except where noted.

<sup>c</sup> Vreeland et al. (2007).

<sup>d</sup> Mormile et al. (2003).

N.S. = Not studied.

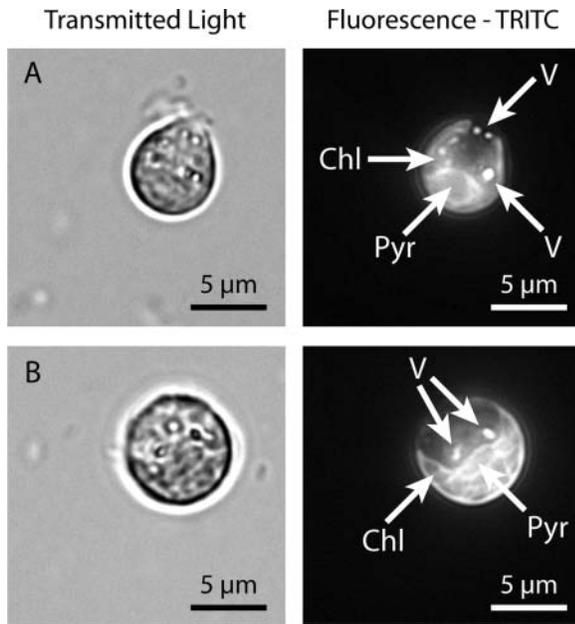


FIG. 5. Black and white photomicrographs of wet mounts showing *Dunaliella* cells from surface brines collected from Saline Valley, October 2005, and observed 28–34 months after collection using transmitted light and epifluorescence microscopy. Arrows show the chloroplast (Chl) and pyrenoid (Pyr). Micron to submicron scale autofluorescent vesicles (V) were observed in the cytoplasm of both *Dunaliella* cells. (A) Pyriform *Dunaliella* cell (TRITC filter; exposure time of 1532 ms). (B) Spherical *Dunaliella* cell (TRITC filter; exposure time of 1828 ms).

the preservation of microorganisms in the Death Valley core is related to the original surface environment.

### $\beta$ -carotene

Cells of *D. salina* and *D. bardawil* accumulate  $\beta$ -carotene in amounts up to 13.8% and 8% of their total dry weight, respectively (Aasen et al. 1969; Ben-Amotz and Avron 1982; Ben-Amotz et al. 1982). Electron micrographs show that  $\beta$ -carotene accumulates in oil globules in the interthylakoid space of the chloroplast (Ben-Amotz et al. 1982). As the  $\beta$ -carotene content of *Dunaliella* cells increases, the pigment spreads throughout the chloroplast (Borowitzka and Siva 2007).  $\beta$ -carotene pro-

TABLE 2  
Medium recipe

Component	GB <sup>a</sup> (grams liter <sup>-1</sup> )
K <sub>2</sub> HPO <sub>4</sub>	0.5
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2.0
KCl	4.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.0
Glycerol	5.0
NaCl	210

<sup>a</sup>GB medium is modified after PGB medium (Schubert et al. in review).

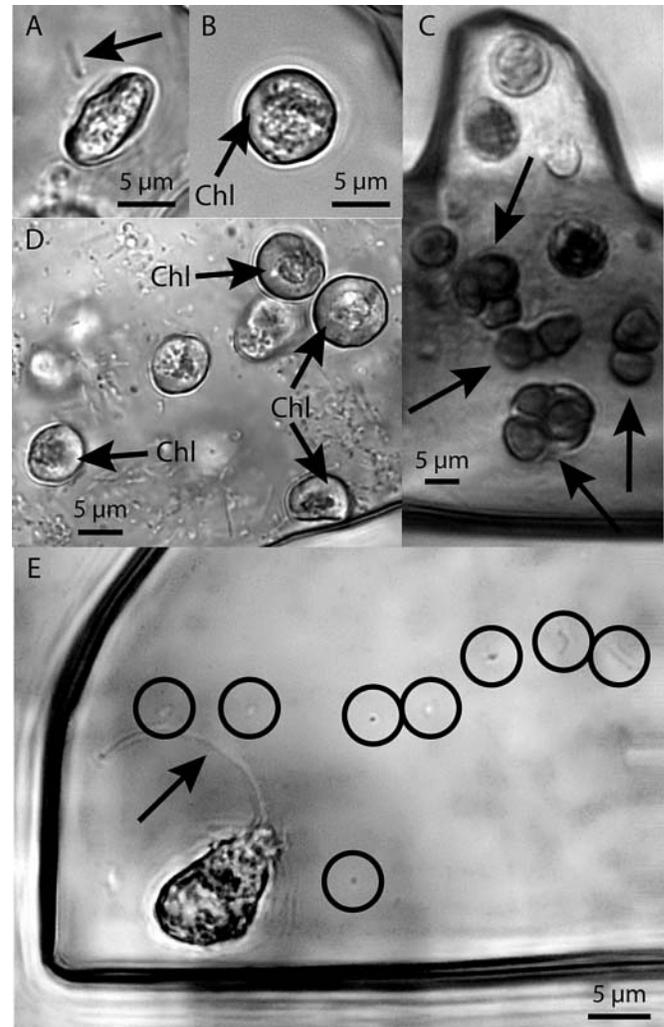


FIG. 6. Black and white photomicrographs of prokaryotes and *Dunaliella* cells in fluid inclusions in halite collected from Saline Valley, October 2005, and observed 28–34 months later. (A) Ellipsoid *Dunaliella* cell with rod-shaped prokaryote cell (arrow). (B) Spherical *Dunaliella* cell with chloroplast (Chl). (C) Clusters of green, mucus-covered *Dunaliella* cells (arrows), possibly in a palmelloid stage, with other spherical and pyriform *Dunaliella* cells. (D) Abundant prokaryotes and *Dunaliella* cells in a large fluid inclusion. Note the chloroplasts (Chl). (E) Pyriform *Dunaliella* cell with a single flagellum (arrow). Note the coccoid and rod-shaped (straight and curved) prokaryote cells (circled) also trapped in the fluid inclusion.

duction by *Dunaliella* cells increases under conditions of high light, high or low temperature, low nutrient, low oxygen, low pH, or high salinity (Ben-Amotz 1996, 1999; Ben-Amotz et al. 1982; Borowitzka and Borowitzka 1989; Del Campo et al. 2007; Loeblich 1982). Orange cells of *D. bardawil* (UTEX LB2538) from desiccated laboratory cultures contain 1–2  $\mu$ m globular orange structures, probably composed of  $\beta$ -carotene, bulging from the plasma membrane (Figure 3F–H). Similar micron-scale structures have been observed bulging from *D. bioculata* cells after exposure to hyperosmotic salt stress (Bérubé et al. 1999).

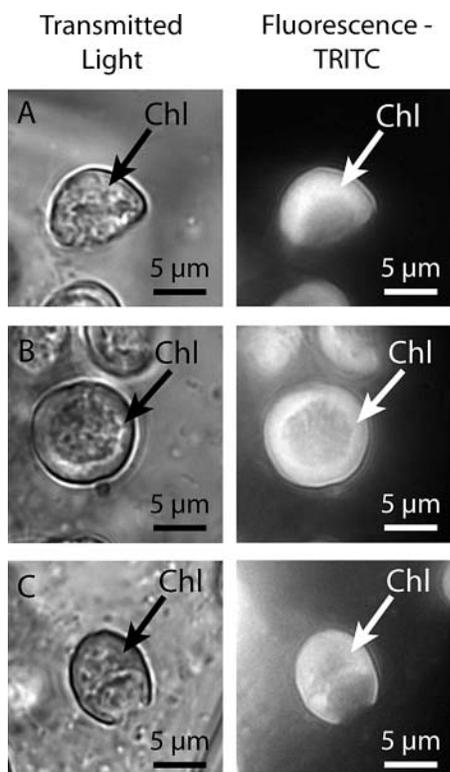


FIG. 7. Black and white photomicrographs of *Dunaliella* cells in fluid inclusions in halite collected from Saline Valley, October 2005, and observed 28–34 months later using transmitted light and epifluorescence microscopy. The chloroplast (Chl) autofluoresces brightly in each cell. The apparent shape of the chloroplast varies on the basis of the orientation of the cell. (A) *Dunaliella* cell oriented with the cup-shaped chloroplast opening down (TRITC filter; exposure time of 266 milliseconds). (B) *Dunaliella* cell oriented with the cup-shaped chloroplast opening towards the reader (TRITC filter; exposure time of 253 milliseconds). The chloroplast appears donut-shaped. (C) *Dunaliella* cell oriented with the cup-shaped chloroplast opening down (TRITC filter, exposure time of 247 ms).

*D. bardawil* and *D. salina* from desiccated laboratory cultures and *Dunaliella* cells from Saline Valley contained micron-scale, orange, red, and pink crystals within, protruding from, on, and adjacent to the cells (Figure 11A–J). These crystals, interpreted as  $\beta$ -carotene, apparently formed from intracellular materials associated with *Dunaliella* cells. We do not fully understand the processes by which  $\beta$ -carotene crystallizes within and on the surface of *Dunaliella* cells, but the occurrence of  $\beta$ -carotene in globules in modern *Dunaliella* cells suggests that this material is the source of the crystalline  $\beta$ -carotene.

Ben-Amotz (1999) reported that different isomers of  $\beta$ -carotene found in *Dunaliella* cells have different affinities for the liquid or solid (crystalline) state. When concentrated, the 9-*cis* isomer remains oily and difficult to crystallize whereas the all-*trans* isomer easily crystallizes at low temperatures (Ben-Amotz 1999). *In vivo* oily  $\beta$ -carotene globules may therefore contain more of the 9-*cis* isomer than the all-*trans* isomer. We speculate that transformation of liquid to crystalline  $\beta$ -carotene

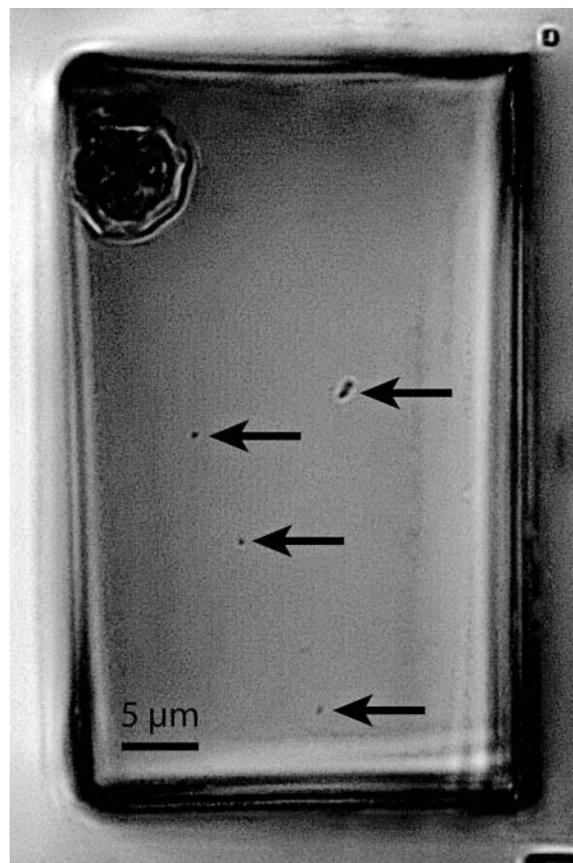


FIG. 8. Black and white photomicrograph of a *Dunaliella* cell and several prokaryote cells in a fluid inclusion in halite from 8.7 m (12,000 years old) in the Death Valley core. Arrows point to one short ( $0.5 \times 1.5 \mu\text{m}$ ), rod-shaped prokaryote cell and three small ( $\sim 0.5 \mu\text{m}$  diameter), coccoid-shaped prokaryote cells.

observed in *Dunaliella* cells trapped in fluid inclusions may be related to the conversion of the 9-*cis* isomer to the all-*trans* form.

Fluid inclusions in halite up to 100,000 years old contained colorless structures, similar in size to the orange globules associated with *D. bardawil* (compare Figures 3F–H and 10D). Globules in fluid inclusions in ancient halite autofluoresced despite lacking observable pigment (Figure 10D), indicating that some of the cellular breakdown products have autofluorescent capabilities.

*Dunaliella* cells in fluid inclusions in halite from the Death Valley core contained orange, red, and pink crystals protruding from, on, and adjacent to the cells, that are similar in color and distribution to the  $\beta$ -carotene crystals observed in modern samples (Figure 11K–P; compare to 11A–J). The antiquity of these crystals required further confirmation that they were  $\beta$ -carotene. Crystal habit, pleochroism, birefringence, chemical composition, solubility in acetone, and melting temperatures of these pigmented crystals, described next, strongly suggested that they were  $\beta$ -carotene.

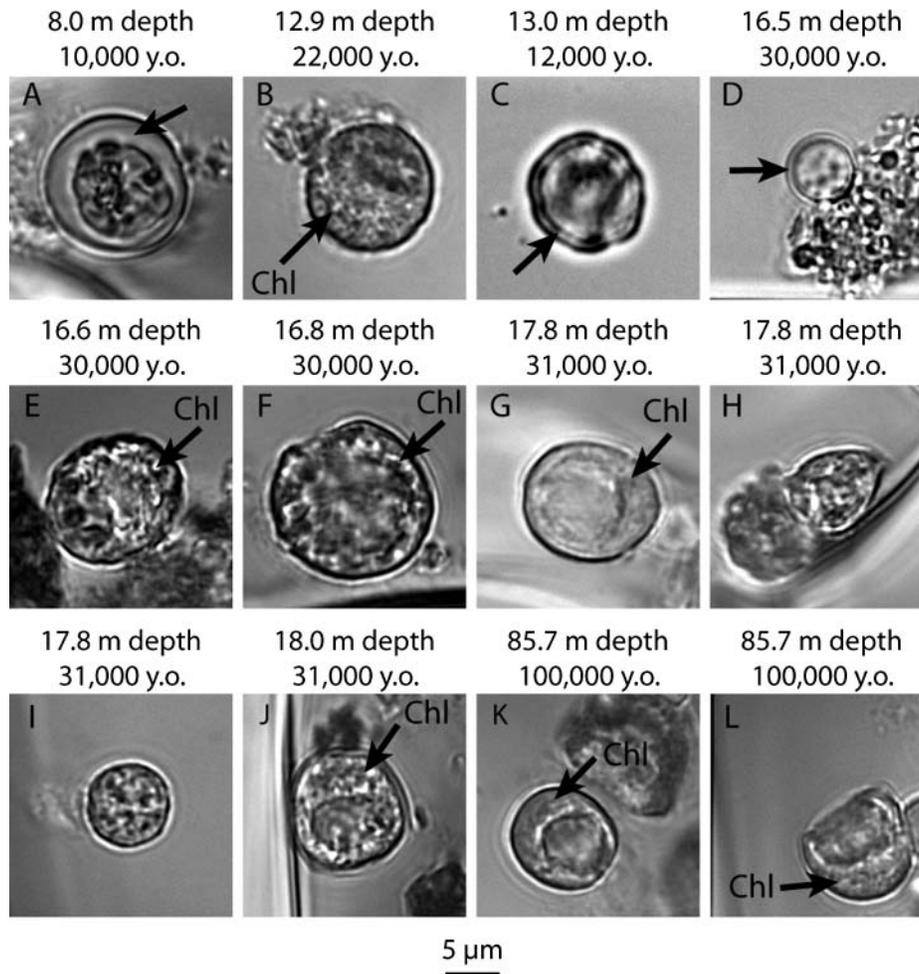


FIG. 9. Black and white photomicrographs of *Dunaliella* cells in fluid inclusions in 10,000-100,000 year old halite, Death Valley core. All *Dunaliella* cells shown were colorless under transmitted light. The depth and age of each sample is labeled above each photomicrograph. Note the excellent preservation and variety of *Dunaliella* cell morphologies. (A) Spherical cell with glycocalyx of non-uniform thickness (arrow). Compare to Figure 4D-E. (B) Spherical cell with chloroplast (Chl). Compare to Figure 4C. (C) Subspherical cell with coat of uniform thickness (arrow). (D) Spherical cell with coat of uniform thickness (arrow). Compare to Figure 4B. (E) Subspherical cell with mottled interior and poorly defined chloroplast (Chl). Compare to Figures 3C and 4H. (F) Subspherical cell, with poorly-defined chloroplast (Chl). (G) Subspherical to pyriform cell with a chloroplast (Chl). Compare to Figure 3A. (H) Pyriform cell lacking distinguishable internal structures. Compare to Figure 3E. (I) Spherical cell lacking distinguishable internal structures. Compare to Figures 3D-E and 4I. (J) Subspherical cell with a chloroplast (Chl). Compare to green cell in Figure 3H. (K) Ovoid cell with chloroplast (Chl). Compare to Figure 7C. (L) Subspherical cell with a chloroplast (Chl). Compare to Figure 7A.

Suspected  $\beta$ -carotene crystals associated with *Dunaliella* cells in ancient fluid inclusions from the Death Valley core had hexagonal, diamond platelet, or acicular (needle-like) habits (Figure 11). Crystals were pleochroic, meaning they changed colors (orange to pink to colorless) when the angle of polarized light was rotated ( $45^\circ$ ,  $90^\circ$ , and  $135^\circ$ , respectively; relative to the c-axis (long axis) of the crystal) (Figure 11Q-S). Pleochroism is a useful characteristic for crystal identification because only biaxial crystals (monoclinic, triclinic, or orthorhombic systems) show pleochroism with three colors (Nesse 2000).

The crystals of interest displayed maximum pleochroism at angles not parallel to crystal axes ( $45^\circ$  and  $135^\circ$ ), which is consistent with monoclinic or triclinic crystal systems

(orthorhombic systems exhibit pleochroism parallel to all crystal axes) (Dana and Ford, 1922). The crystals displayed strong birefringence under cross-polarized light (i.e., they appear bright against the dark background of the isotropic halite host crystal) (Figure 11T). A previous report described  $\beta$ -carotene crystals as hexagonal or diamond shaped monoclinic platelets (Chapman et al. 1967), consistent with our observations.

Additional experiments to confirm the identification of the purported  $\beta$ -carotene crystals in the Death Valley core analyzed elemental composition, solubility in acetone, and melting temperatures. Electron microprobe analyses, designed to determine the elemental composition of the suspected  $\beta$ -carotene,

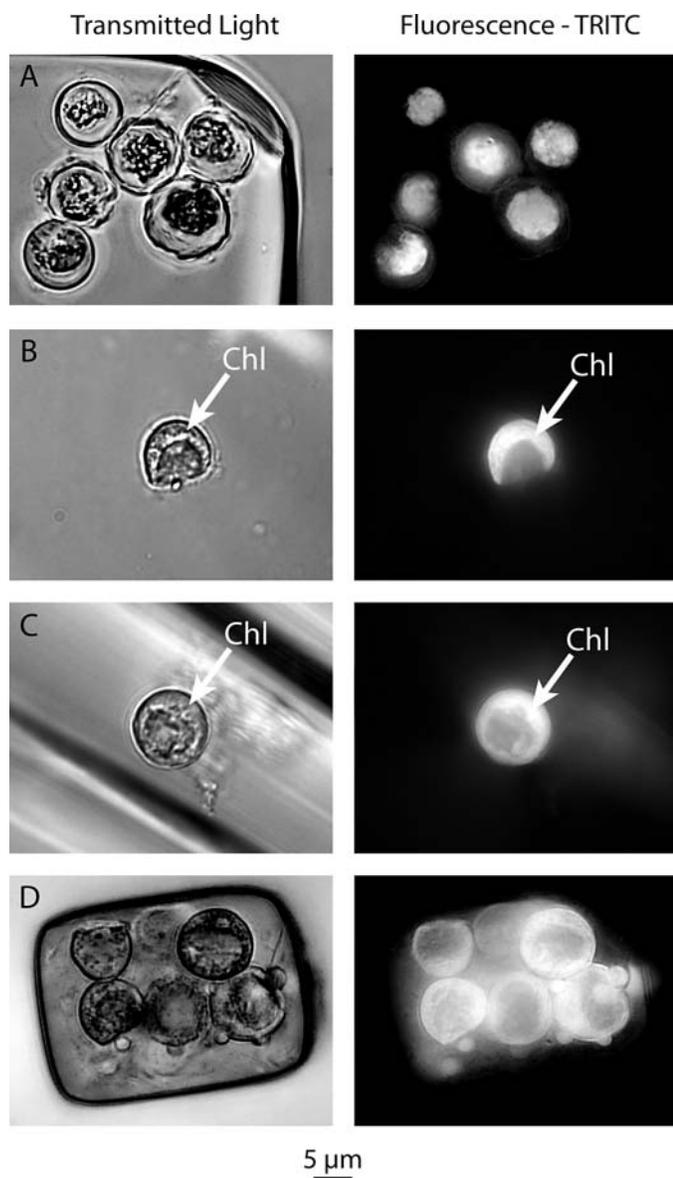


FIG. 10. Black and white photomicrographs of *Dunaliella* cells in fluid inclusions in 12,000-100,000 year old halite from the Death Valley core using transmitted light and epifluorescence microscopy. (A) Spherical to subspherical cells from 8.7 m (12,000 years old). Note that the interiors of the cells autofluoresce more brightly than the glycocalyx (TRITC filter; exposure time of 145 ms). Compare with Figure 4E. (B) *Dunaliella* cell from 12.9 m (22,000 years old) with the autofluorescent cup-shaped chloroplast opening down (TRITC filter; exposure time of 259 ms). Compare with Figure 7A. (C) *Dunaliella* cell from 17.8 m (34,000 years old) with the cup-shaped chloroplast opening out from the page (TRITC filter; exposure time of 134 ms). The chloroplast (Chl) appears donut-shaped and autofluoresces. Compare with Figure 7B. (D) Several *Dunaliella* cells from 85.7 m (100,000 years old) oriented in different directions. The chloroplasts autofluoresce and appear both cup- and donut-shaped. Autofluorescent, micron-scale, colorless, spherical globules are attached to the *Dunaliella* cells. Note that the level of autofluorescence does not decrease with age. Compare with exposure times of modern cells (Figure 7).

failed because the crystals did not produce characteristic x-rays in a range that could be detected by the instrument (see Materials and Methods). Organic crystals, such as  $\beta$ -carotene, are not normally analyzed by electron microprobe because elements of low atomic mass, (i.e., carbon and hydrogen) cannot be identified. Further testing showed that the crystals dissolved in acetone, but not water, which is consistent with the properties of  $\beta$ -carotene (Craft and Soares 1992) (see Materials and Methods).

The melting temperatures of the purported  $\beta$ -carotene crystals in fluid inclusions in halite were determined *in situ* by slowly increasing the temperature of the sample on a heating stage (see Materials and Methods). Crystals melted into liquid droplets at temperatures between 133 and 181°C. The large range of melting temperatures may indicate that several different carotenoid pigments were present, including  $\beta$ -carotene, which melts at 179.8°C (Zscheile and White Jr. 1940). Upon cooling to room-temperature, the liquid droplets did not change shape, form observable crystals, or fade in color which is consistent with previous experiments on  $\beta$ -carotene (Zscheile and White Jr. 1940).

$\beta$ -carotene crystals, despite being sensitive to oxygen (Ben-Amotz 1999) and light (Ben-Amotz et al. 1989), do not show loss of color or any evidence of degradation in fluid inclusions up to 34,000 years old. The breakdown of  $\beta$ -carotene crystals is apparently retarded by (1) the low-oxygen environment of a fluid inclusion, and, (2) removal from sunlight in buried halite deposits.

#### Summary of the State of Preservation of *Dunaliella* Cells in the Death Valley Core

*Dunaliella* cells in fluid inclusions in modern and ancient halite showed no obvious differences in their state of preservation, other than color. This indicates that *Dunaliella* in ancient halite probably occur in the same physical condition as they were when originally trapped in fluid inclusions. The green color of *D. salina* and *D. bardawil* in fluid inclusions from dehydrated laboratory cultures and green *Dunaliella* cells from modern Saline Valley halite indicate that chlorophyll remained in the algae following entrapment in fluid inclusions. Direct evidence for chlorophyll preservation in *Dunaliella* cells in ancient halite from Death Valley includes autofluorescent chloroplasts (Figure 10B–D) and rare, yellow-green colored *Dunaliella* cells (Figure 11M–O). The age of the sample (up to 100,000 years old) apparently did not affect autofluorescence (compare Figure 7 to Figure 10), indicating that some autofluorescent compounds remain, especially in the chloroplast. In the Death Valley core, the presence of intact *Dunaliella* cells that contain  $\beta$ -carotene and chlorophyll strongly suggests that NaCl-rich, low-oxygen, low-light conditions in fluid inclusions in halite retard the degradation of organic matter.

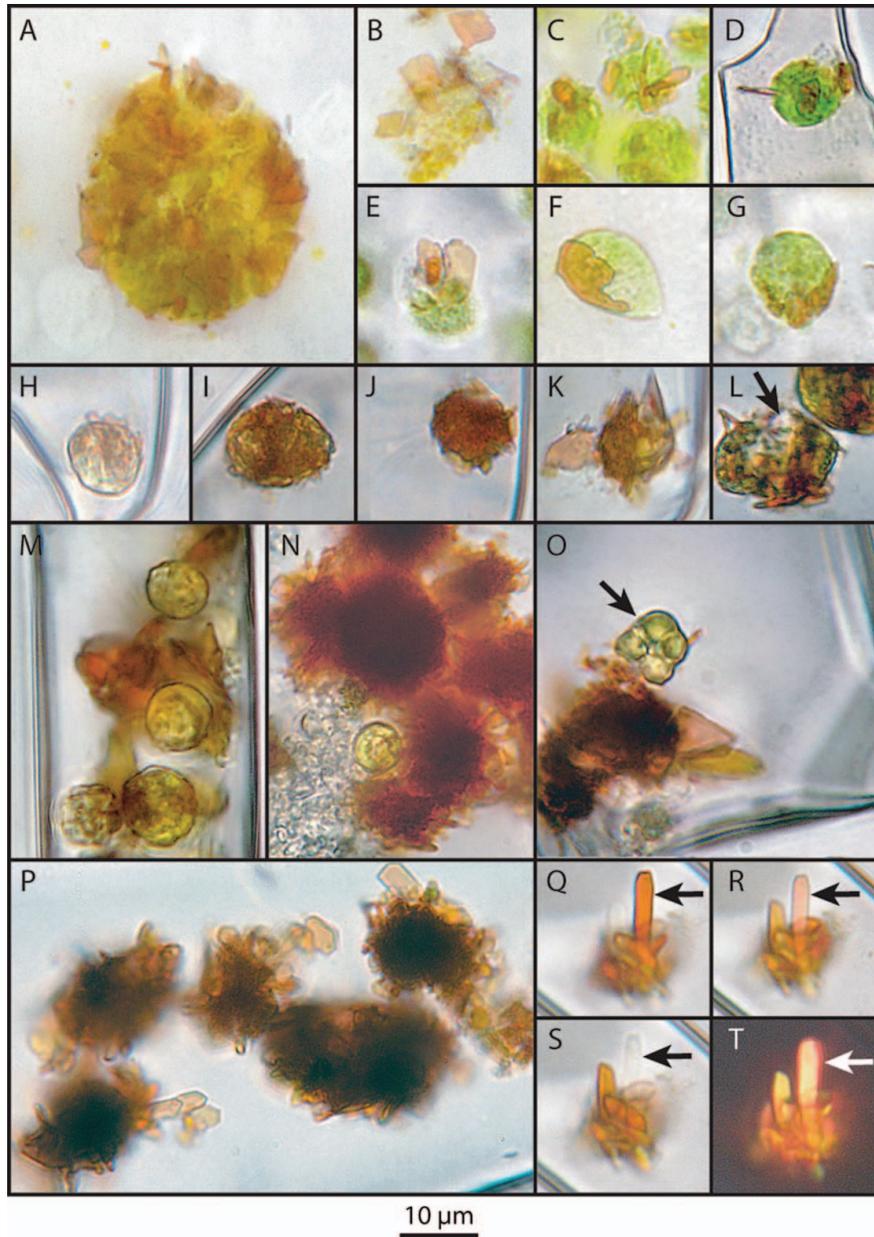


FIG. 11. Color photomicrographs of *Dunaliella* cells and crystalline  $\beta$ -carotene from desiccated uni-algal cultures, environmental samples from Saline Valley, and the Death Valley core. (A-B) Orange and pink  $\beta$ -carotene crystals protruding from *D. bardawil* (UTEX LB2538) cells on the surface of a laboratory-grown halite crystal. (C-G) Orange and pink  $\beta$ -carotene crystals protruding from *D. salina* (Duna001) cells in fluid inclusions in C-E and on the surface of a laboratory-grown halite crystal in F-G. Fluid inclusion walls are outside the field of view in all except D. (H-J)  $\beta$ -carotene crystals on the surface of *Dunaliella* cells in fluid inclusions in 28–34 month old halite crystals from Saline Valley. The abundance of  $\beta$ -carotene crystals increases from H to I to J. (K) Single crystals of  $\beta$ -carotene protruding from a *Dunaliella* cell in a fluid inclusion in halite from 14.5 m (26,000 years old) in the Death Valley core. (L) Two *Dunaliella* cells partially covered with  $\beta$ -carotene crystals in a fluid inclusion in halite from 16.7 m (31,000 years old) in the Death Valley core. Fluid inclusion walls are outside the field of view. The *Dunaliella* cells appear yellow-green. One *Dunaliella* cell appears ruptured (arrow). (M) A fluid inclusion in halite from 17.8 m (34,000 years old) in the Death Valley core with  $\beta$ -carotene crystals and several yellow-orange to yellow-green *Dunaliella* cells. (N) Several  $\beta$ -carotene encrusted *Dunaliella* cells; one non-encrusted, yellow-green *Dunaliella* cell; and several small, colorless, unidentified minerals in a fluid inclusion from 15.7 m (29,000 years old) in the Death Valley core. Fluid inclusion walls are outside the field of view. (O) A cluster of four, green, mucus-covered *Dunaliella* cells (arrow), possibly in a palmelloid stage, with  $\beta$ -carotene encrusted *Dunaliella* in a fluid inclusion in halite from 17.8 m (34,000 years old) in the Death Valley core. Compare *Dunaliella* cells to Figure 6C. (P)  $\beta$ -carotene crystals with hexagonal, platy crystals and outward-radiating, acicular crystals encrusting probable *Dunaliella* cells in fluid inclusion in halite from 14.5 m (26,000 years old) in the Death Valley core. Fluid inclusion walls are outside the field of view. (Q-S)  $\beta$ -carotene crystals in a fluid inclusion in halite from 17.8 m (34,000 years old). Arrow points to the same  $\beta$ -carotene crystal in each figure.  $\beta$ -carotene crystals demonstrate pleochroism in which they change from orange (to pink) to colorless when different angles of polarized light pass through the crystals. Light in Q, R, and S is polarized at  $45^\circ$ ,  $90^\circ$ , and  $135^\circ$  respectively. (T) The same crystals as observed in Q-S, but under cross-polarized light. Note that  $\beta$ -carotene crystals are highly birefringent (bright), relative to the black (isotropic) halite background.

## DISCUSSION

### Long-term Survival of Prokaryotes in Fluid Inclusions in Halite: Significance of *Dunaliella*

When nutrients are depleted, some prokaryotes adapt by maintaining low-level metabolism including DNA repair, and others form endospores, a state in which no metabolic processes occur (Setlow 1995; Johnson et al. 2007). Prokaryotes that maintain low-level metabolism may be better equipped for long-term survival ( $>10^3$  years) than endospores because dormant cells (i.e., endospores) cannot actively repair DNA damage (Johnson et al. 2007). It follows that long-term survival of prokaryotes in a fluid inclusion, which is a sealed system (Goldstein and Reynolds 1994), requires *in situ* energy sources to maintain low-level metabolism.

One potential energy source may be dead prokaryotes in fluid inclusions. Schubert et al. (2009) observed significant numbers of prokaryotes in halite (up to 65 in one fluid inclusion) from specific intervals in the Death Valley core that also yielded cultures (Schubert et al. in review). Viable cells in fluid inclusions may have fed on nutrients provided by dead cells, although the vast majority of the carbon from dead cells may not be available as an energy source for the remaining living cells. Recent research on the use of dead cells as an energy source by *Bacillus subtilis* supports this hypothesis (González-Pastor et al. 2003; Nandy et al. 2007). When nutrients are limited, *B. subtilis* delays sporulation by killing (via cannibalism and predation) co-existing cells and feeding on the released nutrients (González-Pastor et al. 2003; Nandy et al. 2007). More work is needed to determine whether other prokaryotes, specifically those cultured from ancient salt deposits, can maintain metabolic activity by feeding on co-existing cells.

A more likely source of energy for prokaryotes in fluid inclusions is glycerol. It has long been recognized that glycerol accumulated by *Dunaliella* cells may be used by halophilic prokaryotes as an energy source for metabolic processes and growth in saline lakes (Gochnauer et al. 1972; Rodríguez-Valera et al. 1980; Borowitzka 1981; Javor 1984; Rawal et al. 1988; Oren, 1993a, 1993, 1995; Elevi Bardavid et al. 2008; Falb et al. 2008). We have shown that fluid inclusions in halite from 8.1–18.0 and 85.7 m (10,000–34,000 and 100,000 years old) in the Death Valley core contain cells of *Dunaliella* and prokaryotes. Furthermore,  $\beta$ -carotene, which is produced and accumulated by certain species of *Dunaliella* (Aasen et al. 1969; Ben-Amotz 1996, 1999; Ben-Amotz and Avron 1982; Ben-Amotz et al. 1982; Borowitzka and Borowitzka 1989; Del Campo et al. 2007; Loeblich 1982), has leaked from *Dunaliella* cells and crystallized inside fluid inclusions in modern and ancient halite (Figure 11). From these observations, we hypothesize that intracellular glycerol, soluble in water, also leaked from *Dunaliella* cells and served as a carbon source for protracted survival of prokaryotes. Every cultured strain from the Death Valley core (Mormile et al. 2003; Schubert et al. 2009b; Vreeland et al. 2007) originated from intervals that contained

*Dunaliella* cells and prokaryotes *in situ* within fluid inclusions (Table 1).

Confirmation that non-spore-forming prokaryotes trapped in fluid inclusions in ancient halite can metabolize glycerol is provided by two halophilic *Archaea* (*Haloterrigena* spp. DV582A-1 and DV582B-3 from 17.8 m, 34,000 years old) that were successfully cultured in medium containing glycerol as the sole carbon source. These results suggest that ancient *Archaea* buried in halite in Death Valley may have survived *in situ*, for tens of thousands of years, using glycerol. The prokaryotes cultured from the core did not form endospores, indicating they may have been able to maintain metabolic activity to repair DNA damage.

The amount of energy required by prokaryotes to remain metabolically active depends on whether they are actively dividing, maintaining cellular functions other than growth, or only repairing macromolecular (DNA) damage (Price and Sowers 2004). In nutrient-poor environments, some prokaryotes may miniaturize (reduce in size) and limit metabolism to the repair of damaged DNA (Novitsky and Morita 1976; Morita 1982; Amy and Morita 1983; Kjelleberg et al. 1983; Kjelleberg and Hermansson 1984; Norton and Grant 1988; Moyer and Morita 1989; Amy et al. 1993; Bass et al. 1998; Fendrihan and Stan-Lotter 2004). We speculate that miniaturized prokaryotes observed in the Death Valley core from which halophilic *Archaea* were cultured may have used glycerol to survive for long periods of time (Schubert et al. 2009c).

The amount of glycerol required for long-term prokaryote survival may be theoretically calculated. We used an average metabolic rate for repair of macromolecular damage [ $10^{-7}$  hr $^{-1}$  (Price and Sowers 2004)] to calculate the time over which one miniaturized prokaryote cell [10 fg of carbon (Whitman et al. 1998)] could metabolize the carbon in glycerol derived from one *Dunaliella* cell (10  $\mu$ m diameter; 5.5 M glycerol). The result, 12 million years (Schubert et al. 2009c), indicates that prokaryote cells in fluid inclusions in halite from the Death Valley core are not carbon-limited if *Dunaliella* cells co-exist in the same inclusions.

## CONCLUSIONS

*Dunaliella* cells are readily trapped in fluid inclusions in halite. In the Death Valley core, *Dunaliella* cells were observed exclusively in the perennial saline lake interval (8.1–18.0 m, 10,000–34,000 years old) and one layer in a shallow saline lake interval (85.7 m, 100,000 years old), indicating a strong paleoenvironmental control on their distribution. The distribution of *Dunaliella* cells matches that of miniaturized prokaryote cells and halophilic *Archaea* cultured from the core. We hypothesize that glycerol, accumulated by *Dunaliella* cells, may provide energy for prokaryotes to survive and actively repair DNA damage within fluid inclusions for at least 100,000 years.  $\beta$ -carotene crystals, observed in fluid inclusions in modern and ancient halite, provide evidence that intracellular material has leaked from *Dunaliella* cells. Experiments demonstrated that halophilic

*Archaea* cultured from the Death Valley core were able to grow in medium containing glycerol as the sole carbon source.

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